BIOFUEL FROM ALGAE BIOMASS

A PROJECT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

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By

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CERTIFICATE

This is to certify that the research project report entitled **"Biofuel from Algae Biomass"** submitted by **Miss Geetanjali B Hubli** in partial fulfillment of the requirements for the award of the degree of Master of Technology in Biotechnology and Medical engineering with specialization in Biotechnology at the National Institute of Technology, Rourkela is an authentic work carried out by her under my supervision and guidance.

To the best of my knowledge, the matter embodied in the report has not been submitted to any other University/Institute for the award of any Degree or Diploma.

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List of Abbreviation

1. SSTA : Soil surface topping A
2. B2A : Koel river 2 A
3. KAOA: Karnataka original culture A
4. B2D: Koel river 2 D
5. RASA : Rock attached sample A
6. ST2B: Soil Toping 2 B
7. RASD: Rock attached sample D
8. B1B: Koel river 1 B
9. B1A: Koel river 1A
10. B1C: koel river1 C
11. KASC: Karnataka subculture C
12. SSTC: Soil surface toping c
13. KAS1 : Karnataka subculture 1
14. KAS2: Karnataka subculture 2
15. RASB: Rock attached sample B
16. STA: Soil toping A
17. ST2A: Soil toping 2 A
18. RASA : Rock attached sample A
19. TAG: Triacylglycerol
20. FAA : Free fatty acids
21. FAME : Fatty acid methyl ester
22. GHg: Green house gases
23. Mbp : Mega base pair

Abstract

Present research focuses on the study of algae from water sources in and around Rourkela and selection of potential algal species for biofuel production. Algae samples were collected from Koel river at Jhirpani, Lotus point in National Institute of Technology, Rourkela campus and Bramhini river in Rourkela city. Forty different algal axenic culture was isolated from the algal samples, identified the species and Growth kinetics were studied. Among the identified species, *Chlorella Pyrenoidosa, Chroococcus Sp,and Cladophora sp* were found to be potential algae showing high specific growth rate than other species. Furthermore, the highest yield of biomass was obtained with Cladophora Sp and hence it was selected for further study. The combined enzyme and acid pretrement was found to be most effective producing highest amount of reducible sugars which was further converted to ethanol by fermentation with *Aspergillus Niger,T Reesei,Sacharomyces Cerevisiae*. 54 ml of 16 % ethanol was obtained by fermentation process. Further algal lipid was extracted by modified Folch method and biodiesel was produced by transesterification reaction. 0.0583 g/h lipid productivity, 19.6% Lipid yield and 65% biodiesel production were achieved. Thus the study has demonstrated that Chlorella sp. present in local water body of Rourkela is a potential algal species for biodiesel and bioethanol production.

Key words : Biofuel ,Algae biodiesel, Bioethanol, Algae, *Cladophora Sp*, Pretreatment.

INTRODUCTION

2. INTRODUCTION

Algae are photosynthetic single cell organisms, composed of many different types of sugars like mannitol, glucose, starch etc. Some algae are known to have cellulose material in their cell composition. Many algae are known to store oil droplets as their storage food material hence algae are the potential candidates for biofuel like Ethanol and Biodiesel.

The first generation and second generation of biofuels was mainly based on economic production of ethanol and biodiesel from food and oil crops like, sugarcane, sugar cane molasses, palm oil, wheat, rape seed oil, barley, maize etc. (Nigam and Singh, 2010), an increasing debate of food vs fuel aroused by the production of first and second generation of biofuel (Goh and Lee, 2010). Questions on its sustainability also rose due to which scientists started searching for biofuel potential source that could be grown on non-cultivatable land and also by using less water, domestic waste water or sea water.

Algae mass cultivation is highly economic, as algae utilise atmospheric Carbon dioxide and Sunlight, Supplementing of Phosphates and Nitrates additionally is itself sufficient for Algae culture. The water used in algae culture may be repeatedly used numerous times. The harvesting of algae can be done by flotation, sedimentation, filtration, and centrifugation. In this study flocculation method is utilised, by using polyelectrolytes like Potassium Alum and Sodium Hydroxide (Guschina et al 2006)

Utilisation of algae in biofuel production directly contributes to pollution control and nvironmental protection. Algae are capable of absorbing heavy metals from water, metabolise the Phenol, Cyanide and ammonia present in water, Algae can also neutralise Pesticides , They can utilise excess of phosphates and nitrates in domestic waste water. Large amount of Carbon Dioxide fixation and recycling of carbon emission from petroleum fuel combustion could be achieved simultaneously Molecular oxygen is released in air. Therefore Green House effect can be reduced (AL-Rajhia 2012).

The Algae biofuel has great advantage because of their rapid cell growth, requirement of minimum culture area, reduced cost in harvesting and downstream processing, pumping and mixing is possible because of tolerance of shear force (Borowitzka 1992). Extensive research has been done in calculating the area required to cultivate algae for biofuel production. Chisti (2007) utilized the accompanying comparison to gauge the expense of algal oil where it could be a focused substitute for petroleum diesel.

$$\mathbf{C_{algal}\ oil = 25.9 \times 10\text{-}3\ C_{petroleum}}$$

where: C_{algal} oil is the cost of algae oil in dollars per gallon and $C_{petroleum}$ is the cost of crude petroleum in dollars per barrel This mathematical statement assumes that algal oil has approximately 80 percent of the caloric energy value of crude petroleum for instance, with petroleum price $100 for every barrel, algal oil ought to cost close to $2.59 for every gallon so as to be aggressive with petroleum diesel.

Keeping the above in view, there is need of an effort to search potential algae species that are grown in local, regional and global region and to undertake systematic research for its harvesting to produce biofuel. In this study Algae from various water bodies in and around Rourkela has been studied and biomass of algae axenic culture with highest growth rate among all the algae was used to produce biofuels.

Objective of study

The specific objectives of this project is as follows-

- Isolation and identification of highest biomass yielding algae
- Pre-treatment of algal biomass and its optimisation to maximize the production of fermentable polysaccharides from cellulosic and starch component present in algae biomass
- Fermentation of pretreated biomass for ethanol production.
- Biodiesel production by transesterification of algal lipid extracts.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

In year 2008, fossil fuels represented 88% of the worldwide consumption (Brennan and Owende, 2010). The extensive use of fossil fuels has promoted instability and environmental change by expanding greenhouse gas (Ghgs) outflows because of utilization at a higher rate. The consumption of fossil fuels is presently broadly acknowledged as unsustainable because of draining assets and the gathering of Ghgs in the environment that have officially surpassed the ‘‘dangerously high” limit of 450 ppm CO_2 emission (Schenk et al., 2008). With the excess of anthropogenic GHG emissions and draining fossil fuels, primarily because of substantial scale utilization of fossil fuels in transportation. It is very essential to reduce the use of fossil fuels and embrace approaches towards the application sof renewable energy sources which are proficient in sequestering the atmospheric CO_2, to minimize the reliance on fossil fuels and additionally to keep up environmental and financial sustainability (Brennan and Owende 2010)

Biofuels are an appealing option to current petroleum fuel problem as they could be used as transportation fuel with little change to current automobile technology. Biofuels are renewable fuels from organic sources that might be utilized for electricity generation, heat generation, power and fuel. Biofuels could play crucial role in supplementing petroleum based fuels and also reduce CO_2 emission.

Algae are photosynthetic organisms, they are present in single to multicellular form. They are generally found in soggy or wet places and all types of water bodies hence algae are common in aquatic and terrestrial environments (Wagner, 2007). Algae are classified as kelp (macroalgae) and phytoplanktons (microalgae). Most of the algae are eukaryotic except cyanobacteria (earlier known as Blue green algae) (Packer, 2009). Algae are similar to plants because they also require sunlight, water and carbon dioxide for growth. (Bruton et al., 2009). Fresh water algae are algae which can grow in rivers, ponds, stagnant water or even domestic waste water. Marine algae are those which live at middle saline levels and hyper saline

conditions. Marine algae are seen floating on the tides, they are multicellular with defined cell types and tissues containing specialised cells. The motility of marine algae is due to presence of flagella, mostly the gametes have flagella.

The algal biomass contains different types of sugars and lipids in variable quantity. The Neutral lipids (Triacylglycerols) in algae can be extracted to convert into Biodiesel by Transesterification process (Chisti, 2007). Oil content in microalgae could be reach up to 80% of dry biomass based on species used,

Different types of lipids, hydrocarbons, complex oils are produced in algae cells (Banerjee et al., 2002).Algae can grow by fixing environmental carbon dioxide (Rittmann, 2008:Petrou and Pappis, 2009).Algal lipids constitute unsaturated fatty acids of medium-chain (C10–C14), long-chain (C16–C18) and-long-chain. Under starvation and stress conditions lipid level may increase in many folds in the form of triacylglycerol or TAG. Nutrient depletion in media induces lipid accumulation in algae. The limited supplement of nitrogen containing media components prevents cell division of induces slow growth therefore forces the storage of lipids as storage food in existing algae cells which will not divide in this condition. (Meng et al.)It might be rich in proteins or rich in lipids or have an adjusted arrangement of lipids, sugars and proteins. On the premise of insignificant dietary prerequisites the rough sub-atomic equation of the microalga biomass is assessed as Co0.48h1.83n0.11p0.01 (Chisti, 2007).

2.1Mechanism of Neutral Lipid production in algae

Lipids are classified as Fatty acids and their derivatives, Triacylglycerol's, Wax esters, Phospholipids, phosphoglycerides and sphingomyelin, Isoprenoids (based on isoprene structure).The algal lipids that are convertible to biodiesel are neutral Lipids like tryglycerides

and cholesterol. Some polar lipids like phospholipids and galactolipids are synthesised in algae. Three mechanism are found in algae for triacylglycerol syntheses.1Pyruvate formate lyase enzyme mediated lipid syntheses, 2 .Desaturation and elongation of unsaturated lipids, Acetyl Coenzyme A mediated lipid syntheses (Hemschemeier and Happe, 2005). The vicinity of this catalyst in algae permits fermentative conduct when oxygen is low (Woodward et al in 2000) .

The high productivity of in algae species is due to different ATP transesterification path ways and Glucose-6phosphatase pathway. C3 carbon fixing pathways is common pathway observed in most of the algae species but C4 carbon fixation is also observed in algae. Algae on account of their antiquated beginning and single-celled nature, (Reinfelder et al., 2004) showed that marine diatoms have C4 pathways.

Increasing Lipid productivity in algae

Stress induction in algae culture leads to intracellular oil droplet-formation. This fact is a key to increase the lipid productivity. Basillarophyceae also known as Diatoms have Silica on their cell wall, the depletion of silica in diatom culture also induces lipid droplets in its cytoplasm. Stress can be induced by depletion of nitrogen in the medium for higher lipid yield. The genetic modification of diatom by overexpression of carboxylase, which catalyses the biosynthesis of lipids did not produce high lipid productivity (Chisty et al., 1998; Banerjee et al 2002; Hsieh and Wu 2009).

Biodiesel syntheses by Transesterification of Algal lipids.

Transesterification is reaction between fatty acid or ester molecule and alcohol, where organic R group of ester and R’ group of alcohol is exchanged. Algal lipids have viscosity higher than diesel viscosity, transesterification reduces the original viscosity of algae lipids. Ethanol, Amyl

alcohol Methanol, Propanol, Butanol are used for this reaction. Methanol is widely used because its low cost.

This reaction is carried out in presence of an acid or base catalyst. Acids can catalyse reaction but rate of conversation is extremely slow. Base catalyst mediated transesterification is 4000 times faster than acid transesterification. The base catalyst have higher reaction rate for transesterification of TAG (Huang GuanHua et al 2010) therefore they are used in commercial production of biodiesel , during base catalysed reaction soap is formed and catalyst is lost in the reaction .Additional catalyst must be added to compensate loss of catalyst in soap formation.

In the reaction mixture of transesterification reaction , if free fatty acids (FAA) are more than five percent ,the soap present in transesterification reaction is will inhibit separation of biodiesel and glycerol and emulsion is formed during water wash. Glycerine and biodiesel separates when transesterification is completed.

Lipase enzyme is also used for transesterification reaction but compared to base catalysed reaction, enzyme transesterification is slow. Enzymes show good tolerance to FAA level. Since enzymes are expensive and degree of reaction is low, enzyme mediated reaction is not a preferred method for biodiesl production (chisti 2007;Fuls J 1984; canakci et al 1999;fukuda et al 2001).

2.2Imports and prices of Crude Oil:

In the year 2012-13 India imported 184.795 MMT petroleum of 7, 84,652 crore. The import of petroleum increased by 7.61 % in 2012.Due to fall in Rupee value the cost increased by 16.73% In US dollars the increase in petroleum crude oil import was increased by 3.30%. The average price of International crude oil (Indian Basket) was US$107.97/bbl. in 2012-13 as compared to US$ 111.89/bbl. in 2011-12.During 2011-2013 rupee depreciated by 12.50% which explains

Increase of India's import bill in terms of rupee. (Indian Petroleum and Natural gas Statistics 2012-2013 Government of India).

2.3 Indian Petroleum and Natural Gas Statistics

25% of oil consumed in India is produced by Indian oil resources and 75% of fuel is imported. The consumption of petroleum in India has increased by 4.92 % in 2012-2013

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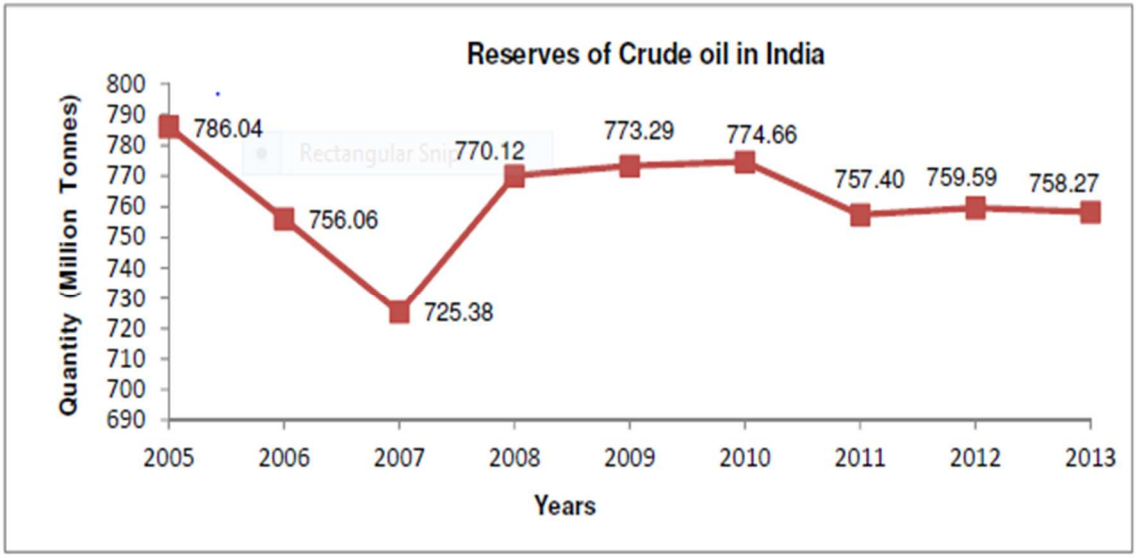

Fig1: Reserves of Crude oil in India

2.4 Diesel

Diesel is a fractional distillate of crude petroleum between 200°C to350 °C at 1 atm pressure. Diesel contains hydrocarbons between 8 to 21 Carbons per molecule. As per European road diesel EN 590 standard minimum certain number of diesel must be 51.The density of diesel must be 0.832 kg/l. 73.25 g/Mj Carbon dioxide emissions. Heat of Combustion is 43.1 MJ/kg. The petroleum crude oil contains paraffinic compounds, Napthenic compounds and aromatic compounds. Alkanes and cycloalkanes groups are present in diesel *(*Chevron1998).

2.5 Biodiesel quality assessment:

- The principle measure of diesel quality is based on its **cetane number**, higher cetane number fuel ignites rapidly, when they are sprayed inside hot compressed air. The high cetane number improves combustion and cold starting. The noise and emission is also reduced by high cetane fuel.
- **Knocking** is the explosion of fuel inside engine.
- **Antiknocking** agents are substances that decreases knocking of fuel , used in high performance engines
- **Octane number** also known as octane rating, it is a standard measure of anti-knock properties and performance of motor and jet fuel. The high octane rate fuels can withstand high compression. Higher octane fuels have high performance.

- **Pour point** of diesel is the lowest temperature at which it form a semi solid fluid and its flow properties are altered. The high pour point indicates higher paraffin content of the fuel. **Kinematic viscosity** is a ratio of the dynamic viscosity and density of a fluid m^2/s.
- **Cloud point** is the temperature at which dissolved solids in diesel starts precipitating
- **Acid value** of diesel is the amount of potassium hydroxide used in neutralising one gram of diesel.
- **Dynamic viscosity** is the measure of resistance to flow (pa-s)

Property	**Limits**
Calcium & Magnesium	5 maximum ppm (µg/g)
Flash Point (closed cup)	93 minimum °C
Methanol Content	0.2 maximum mass %
Water & Sediment	0.05 maximum % vol
Flash Point	130 minimum °C
Kinematic Viscosity	1.9 – 6.0 mm^2/se
Cetane	47 minimum
Cloud Point	°C
Acid Number	0.5 maximum mg KOH/g
Free Glycerin	0.020 maximum % mass
Total Glycerin	0.240 maximum % mass
Phosphorus Content	0.001 maximum % mass
Sulfur S 15 Grade S 500 Grade	0.0015 max. (15) 0.05 max. (500) % mass (ppm) % mass (ppm)
Distillation	360 maximum °C

Table 1: ASTM (American Society for Testing and Materials) D6751 specification of Biodiesel

Reference: http://enterprise.astm.org/filtrexx40.cgi?+REDLINE_PAGES/D6751.htm

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Algae collection from different sources and subculture of algae.

Algal samples were collected in sterile plastic bags from different water bodies in and around the city of Rourkela. Rourkela is one of India's most important industrial cities, it is located in Sundargarh district of western Odisha. The city lies between Latitude 22°25'N and Longitude 84°00'E in the heart of the mineral belt of the state. Different water sources were chosen to collect algae. The sites of collection were Koel River, and Lotus point Pond in National Institute of Technology, Rourkela.

Random grown Algae from a unused glass bottle ,free floating algae from a pond name lotus point from location NIT Rourkela campus was collected and from Koel river of Jhirpani was collected and cultured.

The samples were subcultured using Bolds Basal medium by making media in 80% source water and 20% distilled water. Further subculture it by replacing the media with 60%, 40% 20% and 0% source water and BB media .pH 6.8 and room temperature.

The Bolds basal medium is modified and utilized for fresh water algae culture. To 850 ml dd-H2O stock solution was added and final volume was made up to 1000ml. Stock solution was stored at 4 °c in refrigerator (Guillard, R.r.l and Ryther, J.h. 1962). Stock solutions should not be stored in glass container. Teflon or polycarbonate tube was used to store stock solution. Final pH of medium was set to 6.8 and used for culture. Vitamin B was supplemented by vitamin B tablet, one tablet was used for every 1000ml media.

The figures below show the locations of sample collection .The figure 2 was from Koel River, The sample was collected near the bank of river, where algae was attached to a grass blade. Figure 3 is from lotus point location, algae was collected near the boundary of pond, Figure 4 and 5 are the Rourkela water body and city maps.

Fig2; Algae sample collection from Koel river

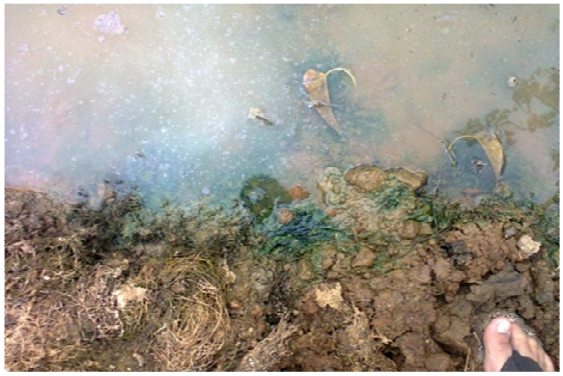

Fig3: Algae Sample collection in Lotus point

Fig4: Rourkela Water bodies Map

Fig5: Rourkela City Map

3.2 Isolation of Algae

The algae subculture was bought to growth phase and serial dilution was performed. 100 µl of algae was inoculated in solid bolds basal medium .The culture plates was incubated at 25^{o}C 24hr light condition was maintained. The subculture of algae grown in first plating was used for further subcultures (Prescott microbiology 2002).

There are several practical challenges in isolation of algae, like fresh algae collected cannot be plated on petri plates because algae would not adopt to its new environment. Therefore only when the fresh algae sample was sub cultured in 100% BB medium , two to three subcultures must be done to bring the cells in growth phase and then they must be cultured on petri plates,.

3.3 Growth Kinetics

The growth Kinetics is used to study several aspects of growth of a microorganism. Every algae cell divides into two daughter cells, the time taken by a population of algae to double is called doubling time and time taken by a single cell to double is called generation time. Growth kinetics was studied by culturing the 18 algal axenic cultures for two weeks .Growth rate was studied by measuring optical density at 600nm and dry weight measurement on every alternative day. Dry weight is measured by centrifuging 1 ml of media, drying the pellets in hot air oven and weighing the algae biomass in centrifuge tube. Algae tend to coagulate and form cluster which could give inappropriate spectrophotometer readings. To prevent errors in measuring growth kinetics, algae culture was kept in orbital shaker to have homogenous cell suspension (Stanbury et al 1997). By following these two methods correlation between optical density and biomass yield per ml for individual algae was obtained and algae with high specific growth rate can be found.

3.4 Identification of Algae

Algae smear was prepared on microscopic slides and observed under microscope. Algae axenic culture was identified by comparing with standard micrographs. Compound Optical Microscope Leica DM -750 Germany attached with ICC 50-HD camera was used for microscopy. Standard monographs of George 1976, Lund, 1960, Belcher and Swale, 1978 was used as reference.

3.5 Batch culture of high yielding algae

Batch culture of B2A sample was grown in five litre erlynmer flask with addition of Bolds Basal Medium. The flask was kept near window side for seven days. Additional medium was not supplemented during batch culture. Then the optical density was measured at the seventh day and algae biomass was collected from medium.

Flocculation was used to collect the biomass. Flocculation method provides effective particle size and easy sedimentation, after flocculation, filtration and centrifuge recovery of algae cell was done (Stanbury et al 1997).

3.6 Fungal culture for fermentation study

Aspergillus Niger is a haploid filamentous algae, it produces citric acid and several enzymes like amylase for starch hydrolyses, Lipase for lipid degradation, and protease for protein digestion, cellulase for hydrolyses of cellulose, lignin, and hemicellulose material to yield glucose for fungal growth. It has a genome size of 3.8 Mbp and 13000 genes. The *Aspergillus Niger* was cultured in Nutrient Broth.

The ***Saccharomyces cerevisiae*** is a yeast, it's known for the production of Ethanol by

Trichoderma Reesei is a filamentous algae, it forms white bolls and develops pale yellow colour colony. It enters steady phase in two days, it has very high growth. This fungi produces several enzymes like cellulase and hemicellulase enzyme Media for this fungal culture must be changed in every alternative days. *S Cerevisiae* was cultured in Potato dextrose agar medium, *Aspergilus Niger* and *T Reesi* were cultured in Nutrient broth. The cultures were preserved in refrigerator in petri plates for further culture (Prescot microbiology 2002)

3.7 Pretreatment optimisation

Preparation of algal hydrolyses: Algaewas ground to fine powder in pestle and marter . Crushed algae was autoclaved at 121 ^{o}C for 15min.For enzyme pretreatment Novozyme celluclast enzyme was used (Ghose K T 1987). Acid pre-treatment: Three ml of 0.1, 0.2 and 0.3 ml of HCl, H2SO4 and Ca (OH) $_2$ was added to 0.1 g of algae and autoclaved. Enzyme pretreatment: Citrate buffer was prepared with 28 g Citric Acid Monohydrate in 100 ml distilled water and its pH was adjusted to 5.5 using NaOH. 20 ml of enzyme in 100 ml buffer.

To 0.1 g of algae powder 3 ml of enzyme buffer was added. Reaction was carried out at 55'C for 2hrs (Ghose K T 1987).

Fungal Pretreatment: *Aspergillus Niger* and *Trichoderma Reesei* produce several enzymes which are capable of hydrolysing the algae biomass. The fungal biomass is harvested and 10% solution was made using distilled water. 3ml of this solution was fermented with 0.1 g algae biomass for 5 days.After the pretreatment test the amount to reducible sugars produced was estimated using DNS method.

DNS method for estimation of reducing sugars

DNS Reagent was made by adding ,1.06 g DNS 1.38 g NaOH, 21g Rochelle salts (Na-K tartarate) in 100 ml water , after all salts are dissolved 0.5 ml phenol was added. Cold water bath was used to facilitate dissolving of salts. Finally 7.6 ml of Phenol was added to increase the intensity of colour (Ghose K T 1987). Glucose Standards: 200 to 1000 µg/ml of glucose standard solution was used.For each standard and test sample 3 ml DNS Reagent was added and incubated in hot water bath for 12 min. Optical density was measured at 540 nm.

3.8 Fermentation and distillation of ethanol

3.81 Fungal Biomass Harvesting *Saccharomyces cerevisiae* and *Aspergillus nige*r and *T. Reesi* was used in this study. The biomass was harvested before fermentation by centrifugation method. The fungus biomass was washed with distilled water for several times and used.

3.8.2 Pretreatment of Algae Biomass

The algae powder remaining after lipid extraction was treated with 100 ml of enzyme citrate buffer at 55 ^{o}C, followed by acid hydrolysis and autoclaved.

3.8.3 Fermentation by *T Reesei,S Cerevisiae, A Niger*

The fungal biomass of *S. cerevisiae, Aspergillus niger* and *T. Reesi* suspended in 50 ml YPD medium and it was inoculated in to autoclaved algae hydrolysates, and fermented for 5 days.

3.8.4 Distillation of Ethanol

After fermentation, the fermentation broth was filtered and distilled at 79^{o}C because ethanol boiling point is 78.37^{o}C (Stanbury et al 1997).

3.8.5 Estimation of Ethanol by potassium Dichromate Method

In this method Chemical oxidation of Ethanol takes place, Ethanol is completely oxidised by potassium dichromate in presence of sulphuric acid .Acetic acid is formed at the end of this reaction. This method is widely accepted and used.

$$2Cr_2O_7^{--} + 3C_2H_5OH + 16H^{+} \text{-----> } 4Cr^{+++} + 3CH_3COOH + 11H_2O$$

During the reaction potassium Dichromate which is yellow colour, was reduced to chromic product and the reduced chromic product had intense green. The intensity of colour change can was measured spectroscopically at 540 nm wavelength. Acetic acid was end product of this reaction (Williams et al 1950).s-diphenylcarbazide was added to stabilise the intense green colour at the end of this reaction. Potassium Dichromate reagent was prepared by adding 1g of Potassium dichromate in Solution 100ml 6N Sulphuric acid. s-diphenylcarbazide saturated solution was made with 20 ml ethanol. Absolute ethanol was used for ethanol standard solution preparation.

3.9Algae Lipid Extraction

Modified Folch method was used to extract oil from algae. Algae was ground in pestle and marter.50 g of *Cladophora sp* was weighed and 500 ml of chloroform was added .The conical flask was incubated at 150 rpm for 2 days. The solvent was filtered using what man 42 filter paper 125mm *100 circles. The extracts were water washed several times water was clear (Xu Han et al 2006).

3.10 Total Chloroform soluble solids

5g of algae powder was put in a 50 ml conical flask, 50 ml chloroform was added to it, it was incubated at 150rpm, and 30^{o}C for 3 days incubation. The chloroform was filtered and filtrate was dried on hot plate and total dissolved solids were weighed using weighing balance (Patra, J.K 2011).

3.11Transesterification

The chloroform extract of lipids was heated at 61 ^{o}C for evaporation of chloroform and filtered to remove suspended solids. Methoxide was prepared by adding 2g of NaOH in 110 ml methanol .Jar was labelled as “Danger: Methoxide”. The standard ratio of lipid to methoxide is 500ml of lipid in 110 ml of methoxide, same ratio was used in the study. 18 ml of lipid extract was transesterified. Lipid extract was heated to 100°C and methoxide solution was added and temperature maintained at 55°C for 3 hrs. Precaution: temperature must be maintained below 64°C to prevent methanol evaporation. After transesterification reaction the lid must be carefully removed.

Methanol solubility test: 1 ml of biodiesel was added in methanol, if it is completely soluble in methanol and clear homogenous bright phase appears reaction is incomplete (Fukuda H 2001).

Emulsification: in 1:1 ratio methanol and water on vortexing it, if biodiesel separates from water quickly and water is free from debris then biodiesel is clean.

Precautions: methoxide is flammable, volatile and toxic. Goggles, nitrile gloves and long sleeves must be worn during this experiment. The ventilation of laboratory must be good Work in a well-ventilated area. T*he* Screw capped bottle must be used to prepare the methoxide (Brennan, Owende.2010).

3.12Biodesiel Quality and Combustion Tests

3.12.1 Unwashed fuel: If the phase separation does not occur quickly after transesterification, it indicates that reaction is incomplete or excess soap is formed (Brennan, Owende.2010). **3.12.2Specific gravity**: The specific gravity was measured by weighing 10ml of sample in specific gravity flasks Owende.2010).

3.12.3Clarity: Biodiesel was poured on water. If turbidity appeared in water then biodiesel is impure.

3.12.4 pH- If the biodiesel contains sodium hydroxide its pH would be 9 and if free from sodium Hydroxide pH is expected to be 7.The pH was measured by pH strips. **3.12.5The pour point** was measured by freezing in refrigerator and is periodically monitoring the pourability at different temperatures (Brennan, Owende.2010).

3.13 FTIR of Diesel and Biodiesel

FT-IR stands for Fourier transform infrared spectroscopy. Electric dipole moment is important property of the molecule to study its IR spectrum .The IR spectrum can be measured by transmittance, absorbance, photoconductivity and emission. FTIR efficiently collects spectral data in wide range. In FTIR method any functional group has absorption /transmittance at specific range. FTIR of individual molecules is its unique fingerprint by analyzing the FTIR spectrum it can be concluded whether a certain reaction has completed and presence of a molecule can be detected. Bruker Alpha E model FTIR was used during this study (Tariq Muhammad et al 2011).This technique was used to obtain infra-red spectrum of diesel and biodiesel.

Zinc Celluloid crystal was cleaned with ethanol and the sample was poured on the surface above crystal. The machine was setup in ATR (Attenuated total reflection) mode in which the light incident on sample is reflected back to the detector. Samples of pH below 6 and above 7 were not used to protect the Zinc Celluloid crystal.

3.14 Material balance:

Batch culture Kinetics

The specific growth rate of algae biomass wascalculated by

$$\mu = 1/t \quad \ln(Xfinal/Xinitial)$$

Where $X_{initial}$ was biomass concentration at the beginning of culture (quantity of inoculum) and X_{final} was biomass concentration obtained after harvesting." t" was the time duration of one batch culture run.

Lipid Yield

The quantification of productivity was calculated by the equation:

V = CL/T

where *C*L is lipid quantity of one batch culture," t"is the time duration of one batch culture run.

Biodiesel yield

Y (%) = WL /WDA

Y is biodiesel yield, *W*L and *W*DA are the biodiesel produced and lipid used for trans esterification. (converti Attilo et al 2009)

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION:

4.1 Algae collection from different sources and subculture of algae.

Algae was collected from differ places, in sterile plastic bags, pH was simultaneously measured. The pH was 6.4 in lotus point and 6.8 in Koel River. The collected algae was maintained in BB medium throughout the study.

Subculture of Algae in Bolds Basal medium: The subcultures maintained in BB medium, the algae cultures were green and free from parasite worm and fungal contamination.

Fig 6: Algae Subculture (first four flasks were Lotus point subcultures, nest two was KA sample subcultures, Last three was Koel river algae subcultures).

4.2 Isolation of Algae

The initial trials of culturing algae on petri plates was failed because B Complex Vitamins was not added in in BBM. When the agar concentration was 1.5%, hardly few colonies were appeared to grow on the perti plate and subsequently died within few days. In later studies 1000 ml media was supplemented with 1Bcosul Tablet, the agar concentration was 1%, consistency of media was like a gel rather than a semisolid agar plate. The Root like streaking was most

successful method in the culture. The filamentous algae formed mat like structure on the plates, to inoculate the filamentous algae the part of mat was cut using the micropipette tip and used.

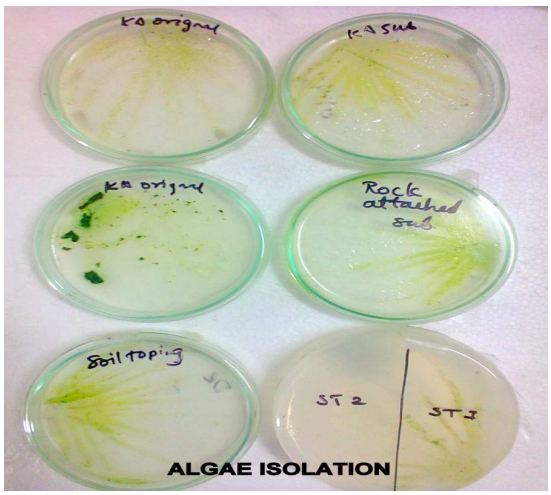

Fig 7 : Fist subculture

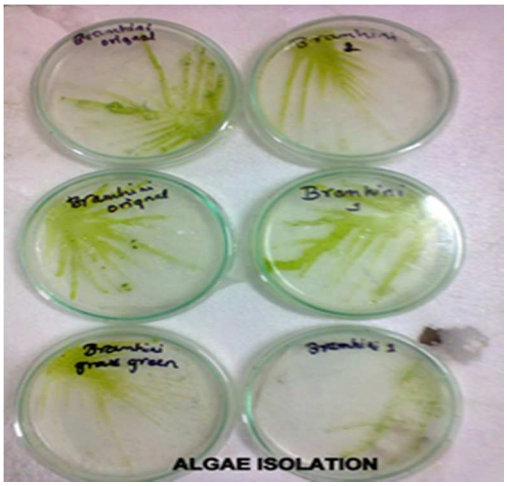

Fig 8 : first subculture

Fig 9: Second Subculture

Fig 10: Third subculture

During first Plate culture Koel river sample was first to appear, the colonies appeared by first three days, eventually on fifth day all the plates showed green colour indicating the growth of algae. In the third subculture the colonies were formed distinctly faraway from each other. The pure colonies of algae was isolated from third subcultures.

From each plate four colonies were picked and streaked on the culture tubes, total of 40 culture tubes were inoculated, within few days some growth was observed in the culture tubes, but only 18 cultures grew and rest of the culture tubes did not show any growth. This could be because the cells which were streaked on culture tubes was not in their growth phase.

Fig 11: Axenic culture Tubes on day of inoculation; fig 12: Axenic culture tubes on 5th day of inoculation

4.3Growth Kinetic study

During this study RASB sample was the first to grow, later B2A and B2D appeared, and some algae samples had extremely low growth. All of soil surface toping a:lgae from the lotus point had pink to red colour pigment excretion in the medium. Hence this set was suitable for study. From the two week study it was observed that B2A and B2D had highest biomass production, though the RASB sample was first one to appear in the flask but biomass was not at expected quantity. From this study B2A sample was having highest growth and biomass yield hence it was chosen for further study to produce biofuel.

Fig 13,14,15: Algae pure culture in BBM for growth evaluation study

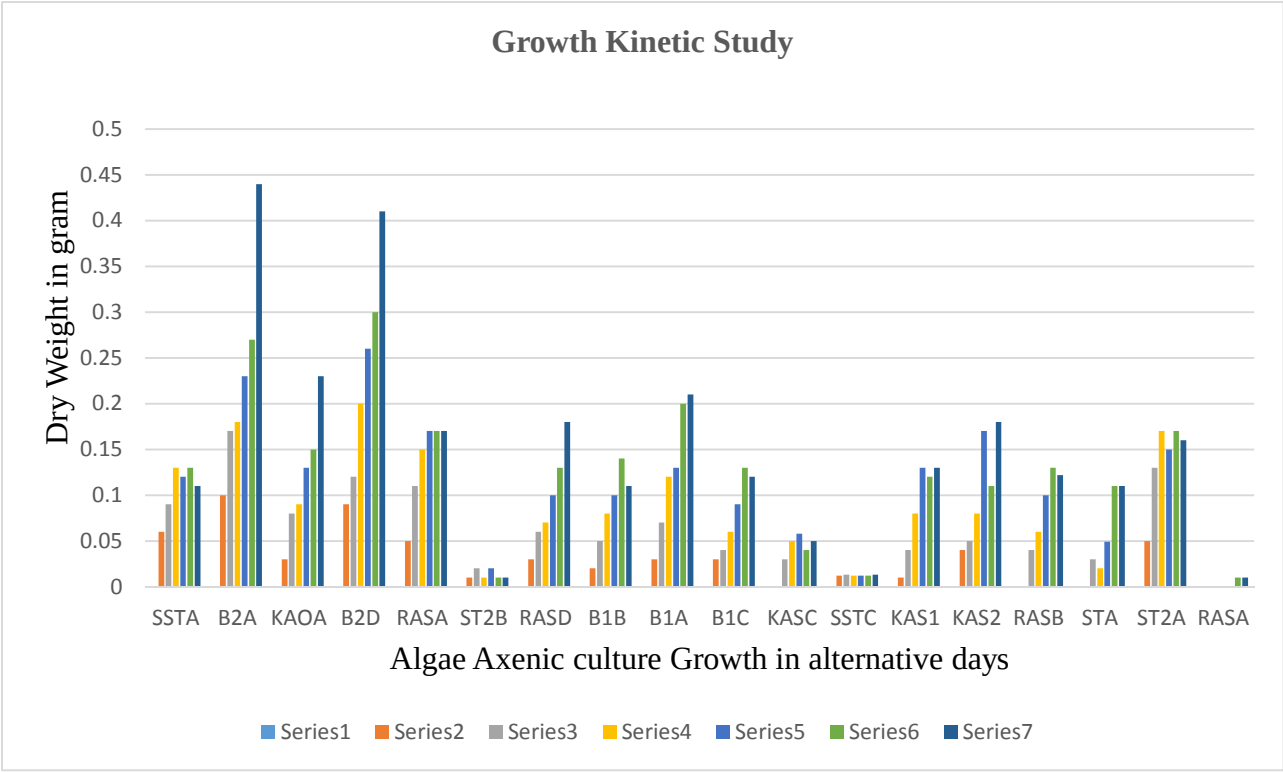

Fig 16: Biomass yield vs Time (Graph showing dry weight of algae in alternative days, from this graph it is understood that ST2B, SSTC, STA ST2A had least growth, these were the sample from lotus point collected above the damp soil. They secreted red colour pigment in the culture and by few days their growth was inhibited, B2A was Koel River sample a filamentous algae with large cells which showed the highest growth.

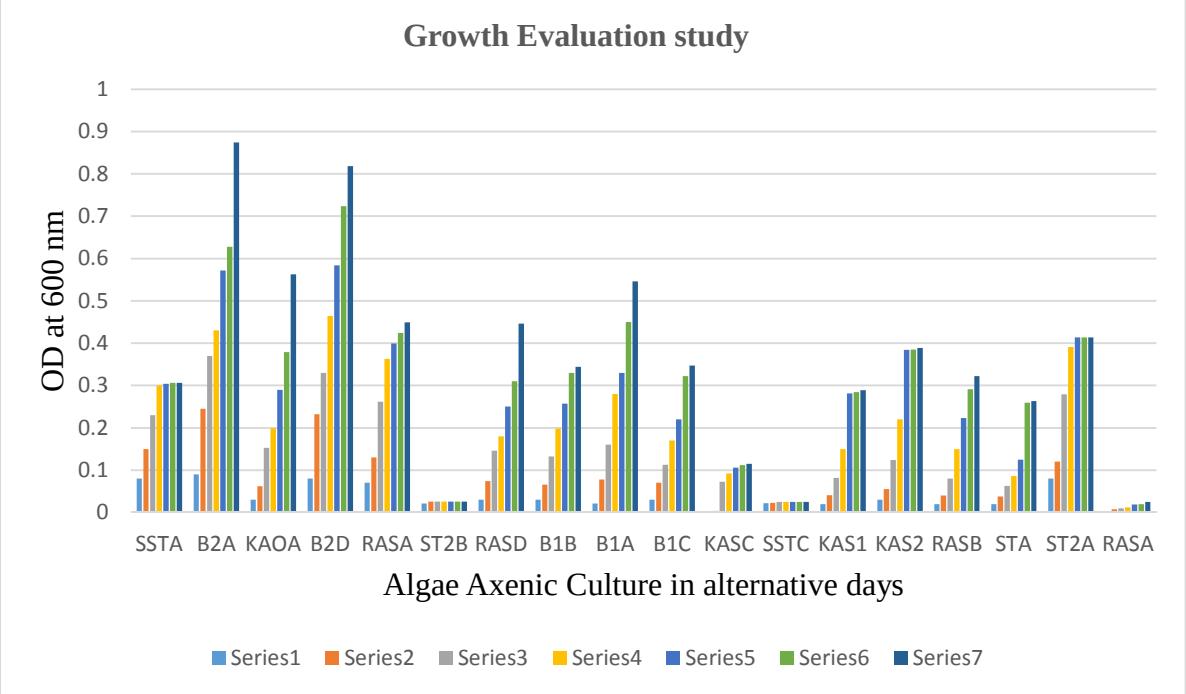

Fig 17: Optical density vs Time (from this graph it is observed that the inoculated algae grew in the BBM after inoculation, in a single batch B2A had highest growth during this batch culture.

4.4 Identification of Algae

B2A was microscopically observed for identification. From the micrograph it was observed that the B2A is a filamentous algae of 50 to 60 µm diameter wide cell, true branching, branching pattern was observed , after every three cells a cell divided to form a new branch. The B2A sample belongs to Plantae Kingdom, chlorophyta division, cladophorales class, from family of Cladophoraceae and Genus *Cladophora*.

The B2A sample was identified as Cladophora Species. *Cladophora* is benthic filamentous macro algae and multicellular. It has highest growth rate among all the isolates because of its size and structure. *Cladophora Sp* are found in fresh water bodies as well as marine water bodies. They can grow up to several meters in length. These species are naturally abundant throughout the world .Blade light green colour of filament, they attached to substrate like any

grass, rock, and bamboo in river by means of rhizoidal cells that extend from filament and float below 5 to 10 cm under water.

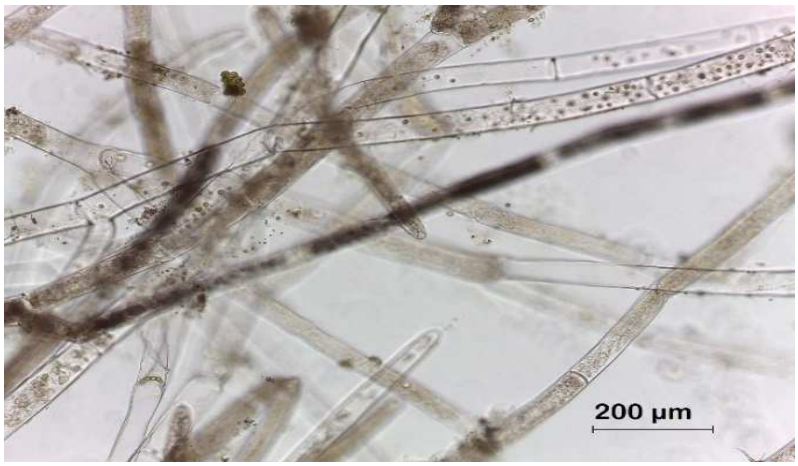

Fig 18: Microscopy of B2A sample in 10X

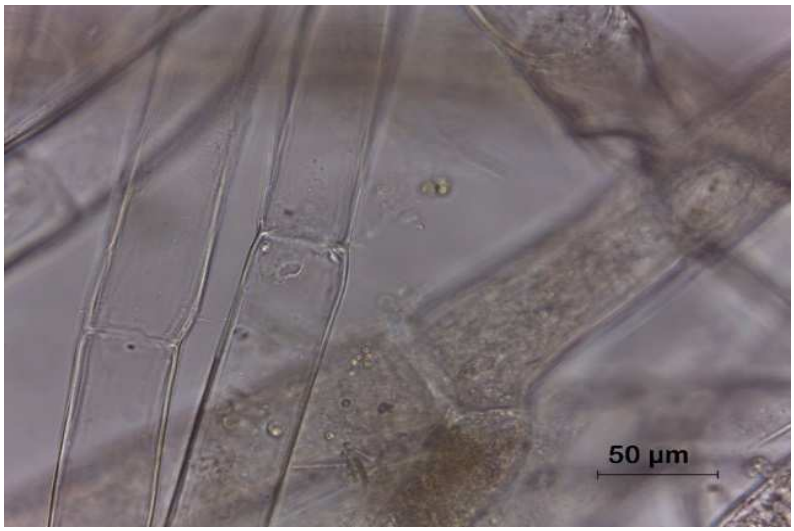

Fig 19 : Microscopy of B2A in 40X

Blades has of 1–3 elongated, clavate cells, forming veins that branch polychotomously, with 3–7 cells at the apex, with blunt connections end-to-end, occasionally forked. Interstitial cells small, regularly arranged, perpendicular to main vein, cortical rhizoids arising from the base of the basal vein cells. Proteinaceous crystalline cell inclusions tetrahedral, non-birefringent, present in the protoplasm between the chloroplasts.`

Cladophora genus has numerous species, they have similar appearance and morphology therefore it is very difficult to differentiate and identify the species of Cladophora by microscopy. The difference in morphology may be due to habitat of filament, water quality, and age of the filament. (Gestinariet al.2010).

Cladophora Algae contain various bioactive constituents comprise of protein, vitamin, minerals and salts, flavonoids, sterol, carbohydrate and volatile components. Phytochemicals present showed potential activities against hypoglycaemic

4.5 Batch culture of high yielding algae and biomass harvesting

Batch culture was carried out in closed culture system of 5L conical flak.

Fig20, 21, 22: Successive Batch cultures of Cladophora Sp Algae

Harvesting of Algae biomass

The cells have certain charge on them, when NaOH or Potassium alum was added, the charges on the cell gets neutralised, which allows it to flocculate on the bottom of container

During flocculation study it was observed that the NaOH of N was sufficient but the increase in quantity of slurry , took very long time for 10 hrs but 1 N Potassium Alum could efficient allow all the cells to flocculate in 4 hrs. Therefore potassium alum is the best choice for the

flocculation of algae biomass. When the NaOH and Potassium Alum was added in the algal slurry, they neutralise the electrical double layer surrounding the algae cells allowing them to flocculate and sediment at the bottom of the vessel

During NaOH flocculation the heavier particles settled easily but the lighter particles formed another phase above the heavier phase, this could be because the charge supplied by NaOH could not have been sufficient to neutralise the charge on them and coagulate, The potassium alum was very successful in flocculation process as it completely clarified the media without forming any light or heavy phase. This is because of multiple ions present in one molecule of the potassium alum, which make it more efficient in flocculating the algal cells and concentrating the biomass.

Though biomass is sediment on the bottom of vessel, but yet it is not free from the water. If the cells are not immediately recovered after the flocculation process the cells turn colourless in three days indicating that the polyelectrolyte greatly effects the cell life. After the cells settle down the water must be drained and the cells must be centrifuge, later washed repeatedly washed with water to remove any traces of the electrolyte which may interfere in further processes. Later cells must be dried in Hot air oven to prevent the invasion of fungus on algae and cells become preserve able.

The specific growth rate was calculated.Consider μx to be the specific growth rate of algae in the batch culture, then

$$\mu x = dx/dt$$

Where x is concentration of algae biomass, t= time in hours, μ is specific growth rate of algae per hours, Therefore μx = 12 g/ (7*24) hr.

μx = 12/168 =0.071 per hours

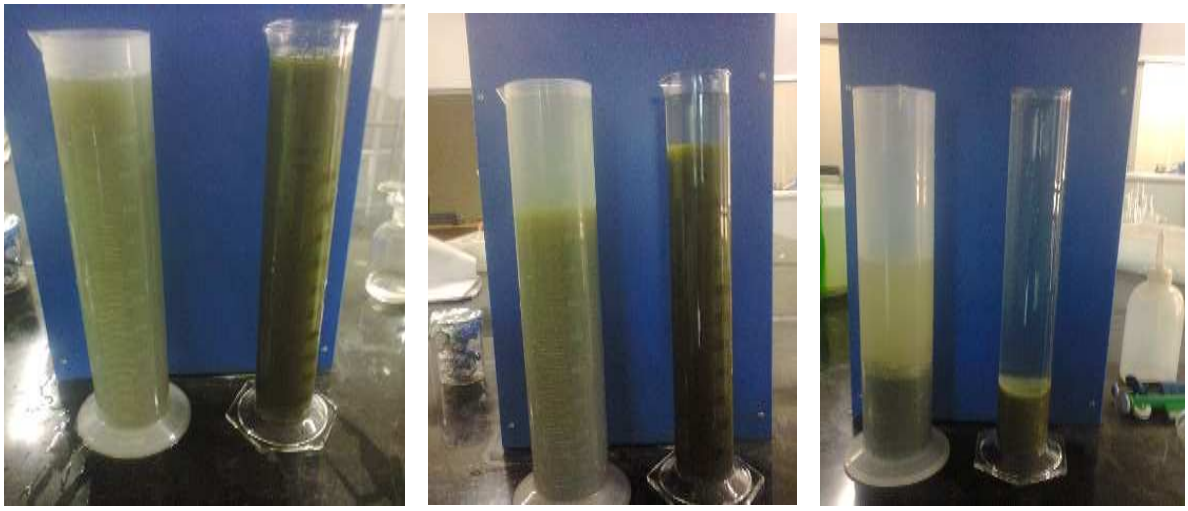

Fig 23, 24,25: Flocculation of algae by 1N Potassium alum and 1N Potassium Hydroxide at the interval of 1, 2, 3 hours.

4.6 Fungus Culture :

The *T Reesei, A Niger, S Cerevisiae* was cultured in nutrient broth and YPD medim. T Reesei formed the mat on the suface of conical flask on the second day of culture, Aspergillus Niger gradually developed, sufficient biomass was obtained..

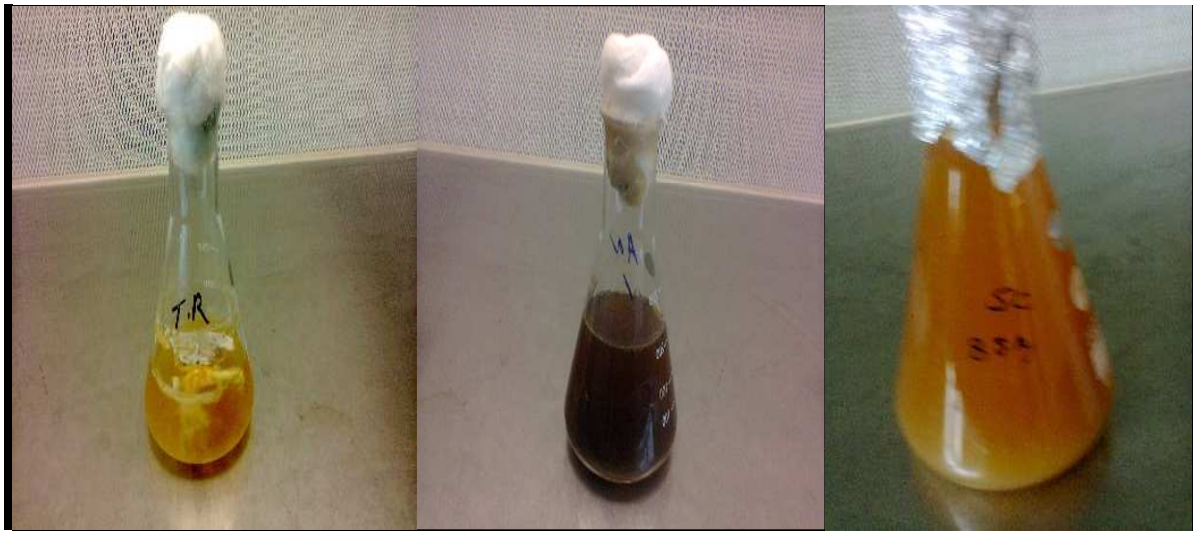

Fig 26 : flask culturing of fungus *T Reesei, A Niger, S Cerevisiae*

Fig 27,28,29: Petri plate culturing of fungus 26.T Reesei,27 A Niger,28 S Cerevisiae

4.7 Pretratment study

Acid pretreatment: 0.2N HCl gave maximum saccharification of 857µg/50 µl.The Calcium hydroxide pretreatment did not saccharify at low concentration but at 0.3N it was considerably high compared to other pretreatments.Sulphuric acid pretreatment was observed to linearly increase with increase in its concentration , but only 0.2 N HCl gave highest amount of saccharification of 0.05mg/0.1g algae biomass.

Enzyme pretreatment:In Novozye pretreatment , saccharification was observed, indicating that cellulose material was present in Cladophora algae.

Fungal pretreatment: The reducible sugars released vere comparitively very lass compared to all other acid hydrolysys ,this may be due to limited substrate For the fungus ,and uptake of reducible sugars by fungus.

Acid and Enzyme, Enzyme and acid pretratment : This study showed that if enzyme hydrolyses was carried out before the acid hydrolyses then the quantity of sugar released was very high. This may be because, after acid hydrolyses the algae biomass is acidic and optimum 5.5 pH for cellulase activity was not obtained as sulphuric acid was not removed when enzyme

was added.When enzyme hydrolyses is done first the enzme has all its functional environment and hydrolsis was sucessful.In this study it was observed that the enzymatic pretratment followed by acid pretratment is optimum for algae biomass pretreatment before fermentation.

Boiling pretreatment: On simple boiling of algae biomass in water (autoclaving) it was observed that supernatent had some reducible sugars in it. This test indicated that the polysaccharides present in *Cladophora sp* algae are not water only on applying optimal pretreatment methods the reducible sugars was available for fermentation .

fig 30 : DNS test for glucose standard curve

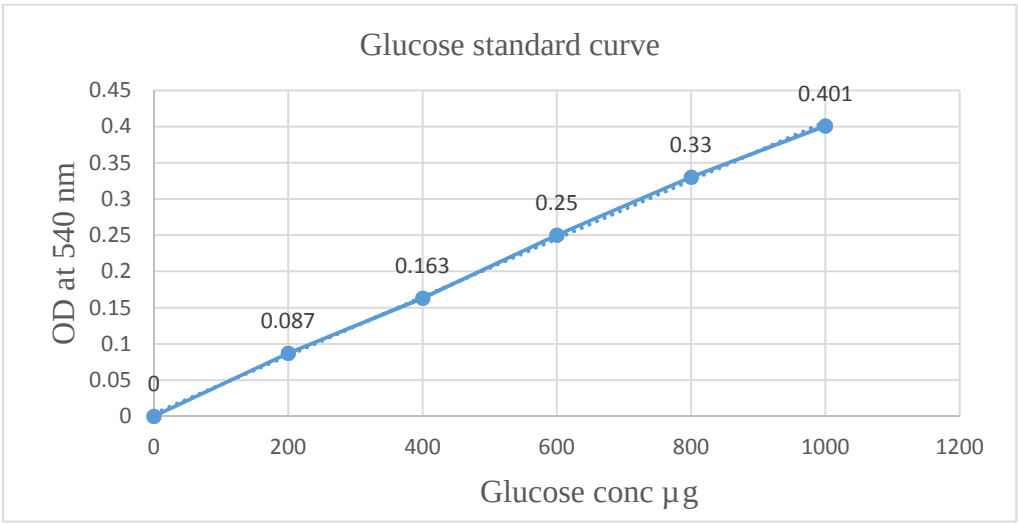

Fig 31: Glucose standard curve graph(In this graph it is observed that the there was linear increase in absorbance as the glucose concentration was increase)

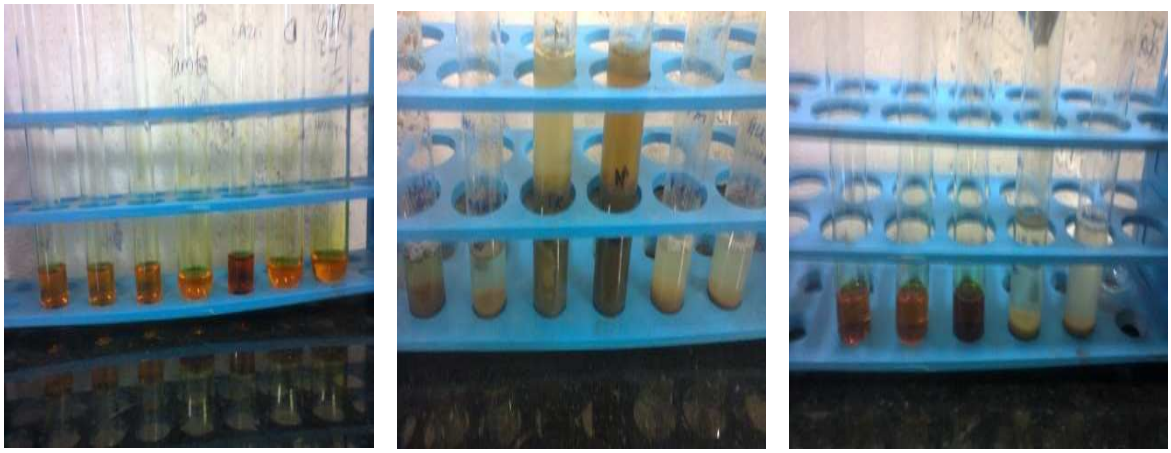

Fig 32,32,34:Acid pretreatment ,fungal and enzyme pretraetment, Acid and enzyme pretreatment

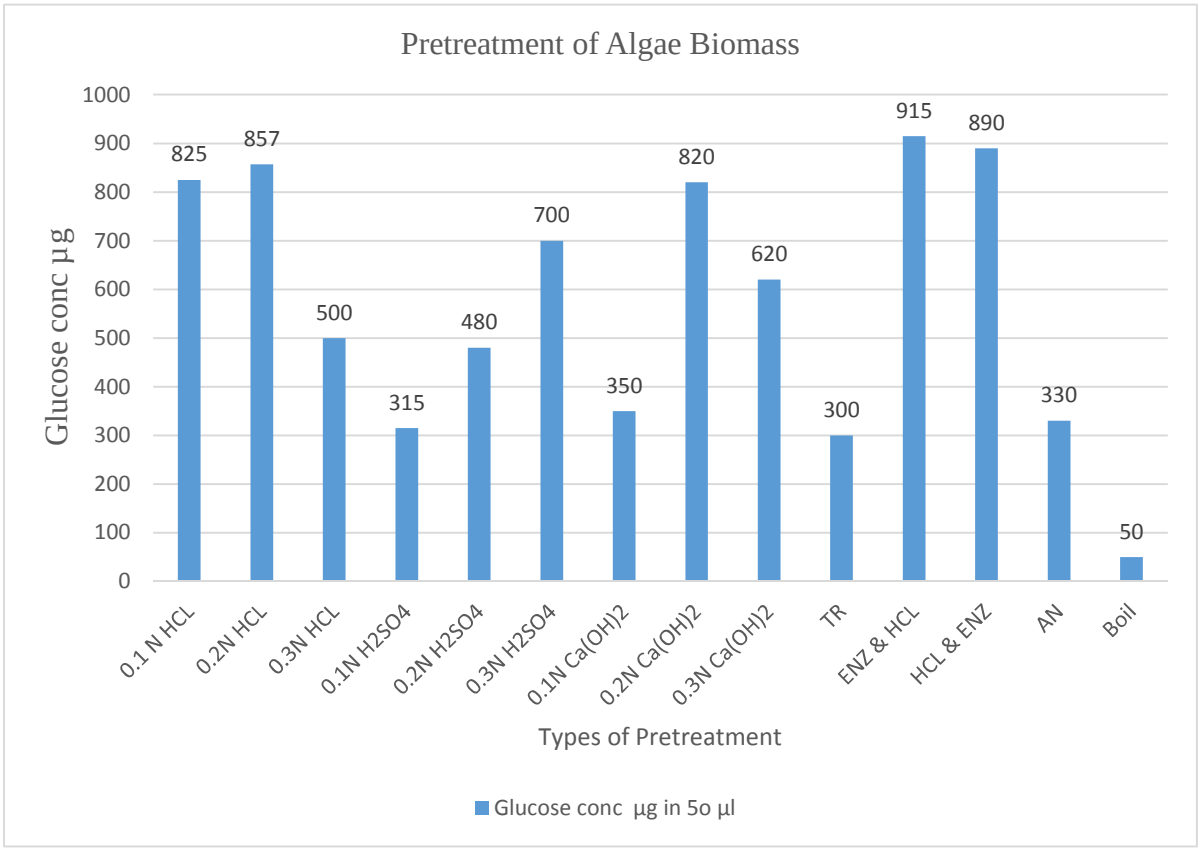

Fig 35: Pretratment saccharification results of Algae biomass.(The graph shows effect of different pretreatments on algae biomass, the amount of sugar released from each methods

4.8 Fermentation and Distillation

The fermentation broth had pH 7 on day 1, on day 2 *T Reesei and A Niger spores* appeared in the form of ball like structures. The broth had sweet smell because of *S Cerevisiae fermentation.* The pH decreased from 7 to 6 on 7th day of fermentation, indicating that ethanol has formed in the broth. By distillation the fermentation broth 54 ml of ethanol distillate was produced. By potassium dichromate assay it was found that ethanol produced was of 16% co

Fig 36 : Fermentation of Ethanol

Fig 37 : Distillation of Ethano

Fig 38,39,40: fungal biomass of *T Reesei, S Cervisiae, and A Niger*

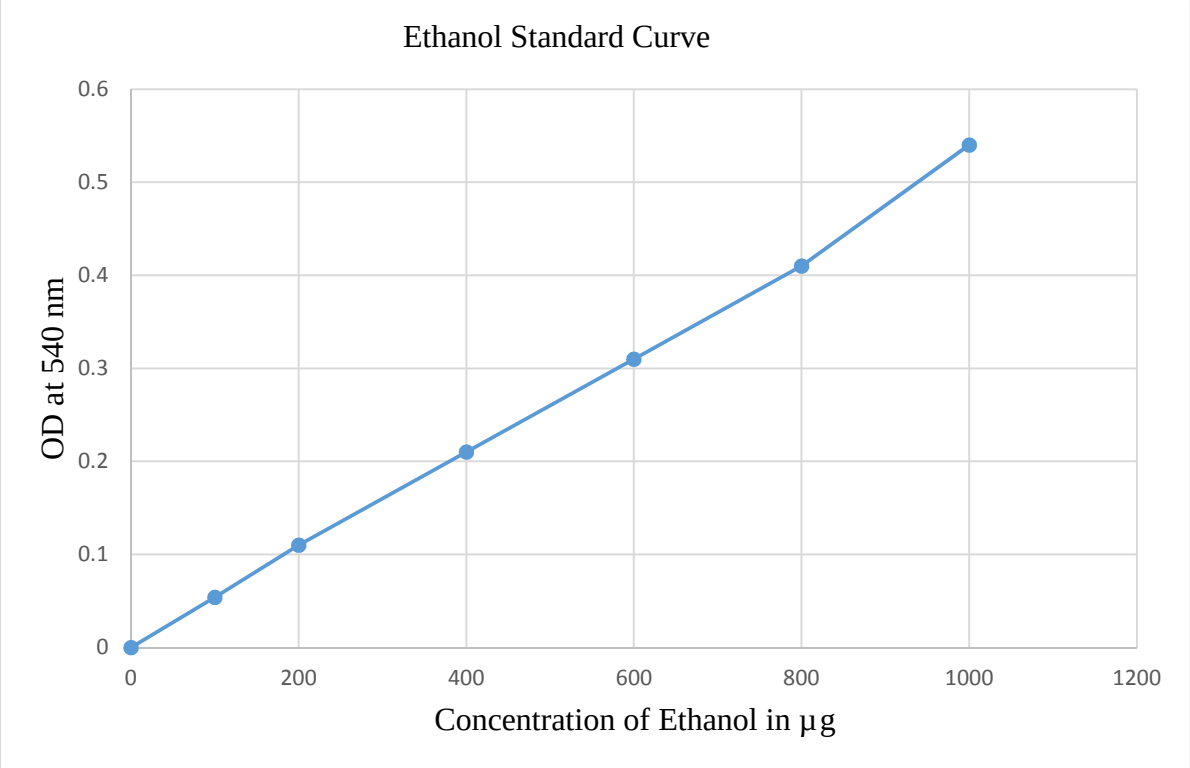

Fig41: Ethanol standard curve graph

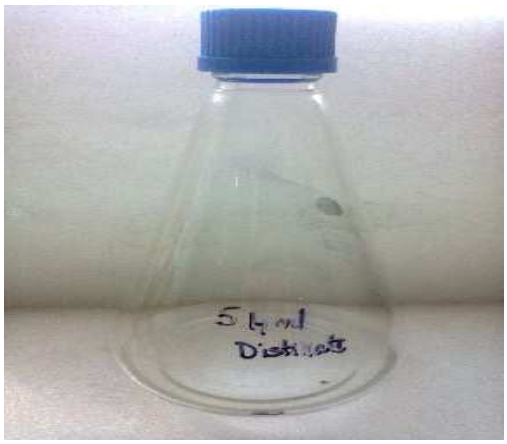

Fig 42: Ethanol distillate

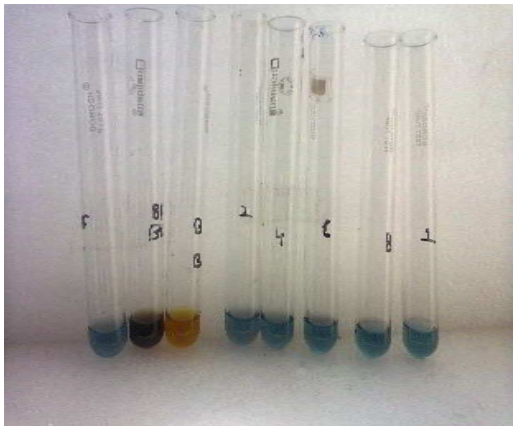

fig 43: Dichromate test

4.9 Oil Extraction

The *Cladophora sp* algae on chloroform extraction ,there was sucessful extraction of lipids, which was confirmed by weighing initial and final biomass during chloroform extraction. After three days incubation for lipid extraction the extract turned dark green,due to excess amount of chloroform soluble solids preent in the extrat.The extract had to washed seveiral times to

remove water soluble matter and the filtrate was heated at 64 ^{o}C to evaporate excess chloroform and it was filtered several times before futher experiments. The chloroform mediated lipid extraction was a successful method to extract lipids 11.2 g of lipids was extracted from 50 g of *Cladophora sp* algae.

Fig 44,45,46 describes the extraction of lipids from algae, chloroform addition and filtration, drying of chloroform and further water washing and filtration.

4.10 Total soluble solids in Chloroform extract

Through this experiment 0.028g of solid material was obtained from 5g algal chloroform extract, which corresponds to 1.4 g of algae solids in lipid extract. Hence the oil extracted from algae contributes 19.6% of total algal biomass.

Fig 47: Extraction and recovering of solids from chloroform extracts of algae

4.11Transesterification

During the transesterification reaction all the components turned to orange colour .After three hours of reaction when the reaction mixture was allowed to settle, distinct layers was observed, which had dark orange colour settled in bottom and light yellow colour above all the layers. Some amount of white precipitation was also observed at the bottom of beaker. The white precipitation was caused by formation of soap. 0.4g of soap ,2 g glycerol and 6.37 g of biodiesiel was produced from the reaction.

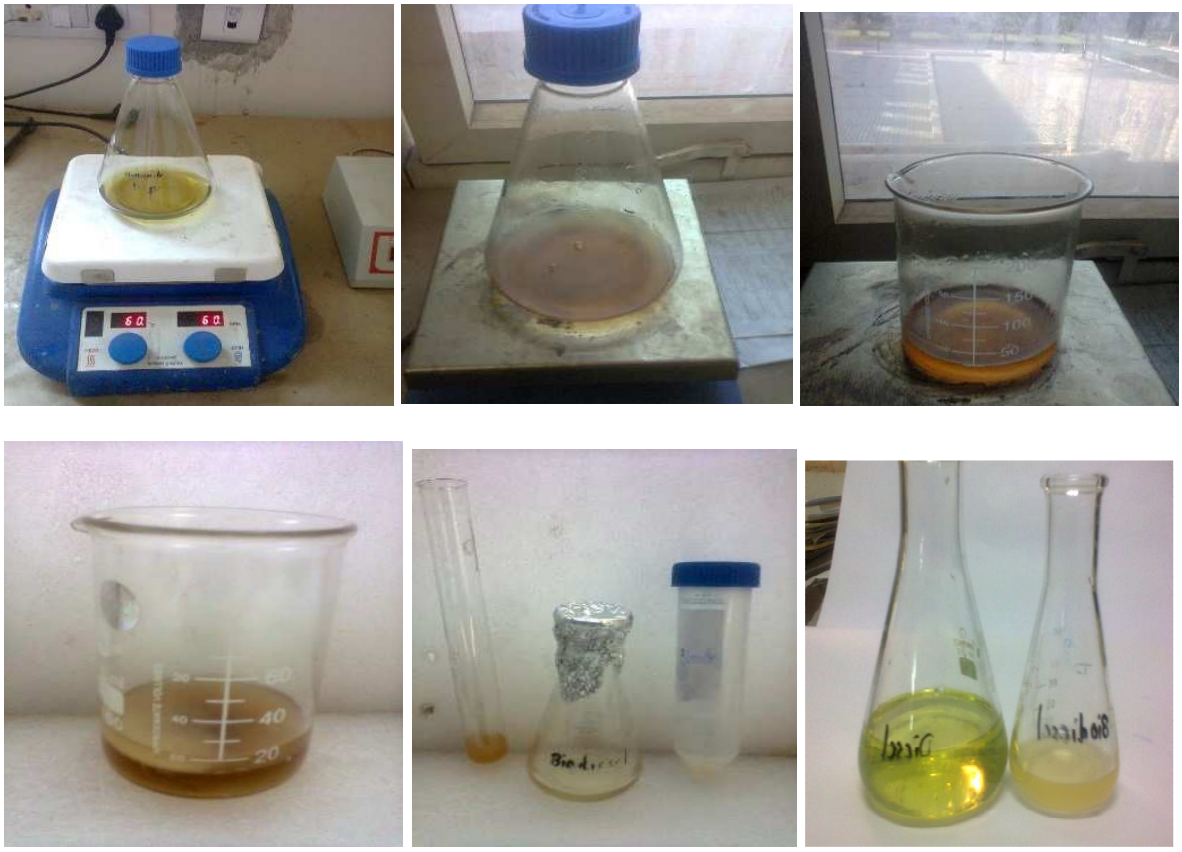

Fig 48 to 53: describe the pocess of transesterification where , lipid extract is washed from the water and filtered several times and dried at 61 C,further heated to 110 degree celcius and methoxide is added, 43 image shows transesterification of lipids by changing into orange colour,44,45 show that the after 3 hours on cooling the biodiesiel separates from glycerol and impurities, 46 shoows the products recovered from transesterification process, fig 47 shows visul difference between algae biodiesiel and Petrolium diesiel.

4.12 Biodiesel Quality and Comburstion Test

Cotton boll flame test: The cotton ball soaked with 0.7ml of biodiesiel burnt for 4 min

Steel rod Flame test: The biodiesiel caught flame between 6-7 sec ,and petrolium diesiel caught fire in 4 seconds.

Pour Point and freeze point test:The biodiesiel is pourable at 7 ^{o}C and freezes at 2 ^{o}C

Clarity test: After several washes biodiesiel formed clear zone with water indicating that it has very lesst content NaOH and Glycerine in it .

Specific grvaity measurement: Ethanol 12.06g (16%) ,Biodiesiel 10.82g,Diesiel 9.16 g

Fig 54: Cotton ball test

Fig 55: Steel rod flame test

Fig 56: Pour point and freeze point test

Fig 57: Clarity test



Fig: 58 Biodiesel specific gravity; Fig 59: Ethanol specific gravity; Fig 60 Diesel Specific gravity.

4.13 FTIR of Diesel and Biodiesel

From the FTIR spectrum analysis of diesel and biodiesel, transmittance peaks appeared in various distinct regions.

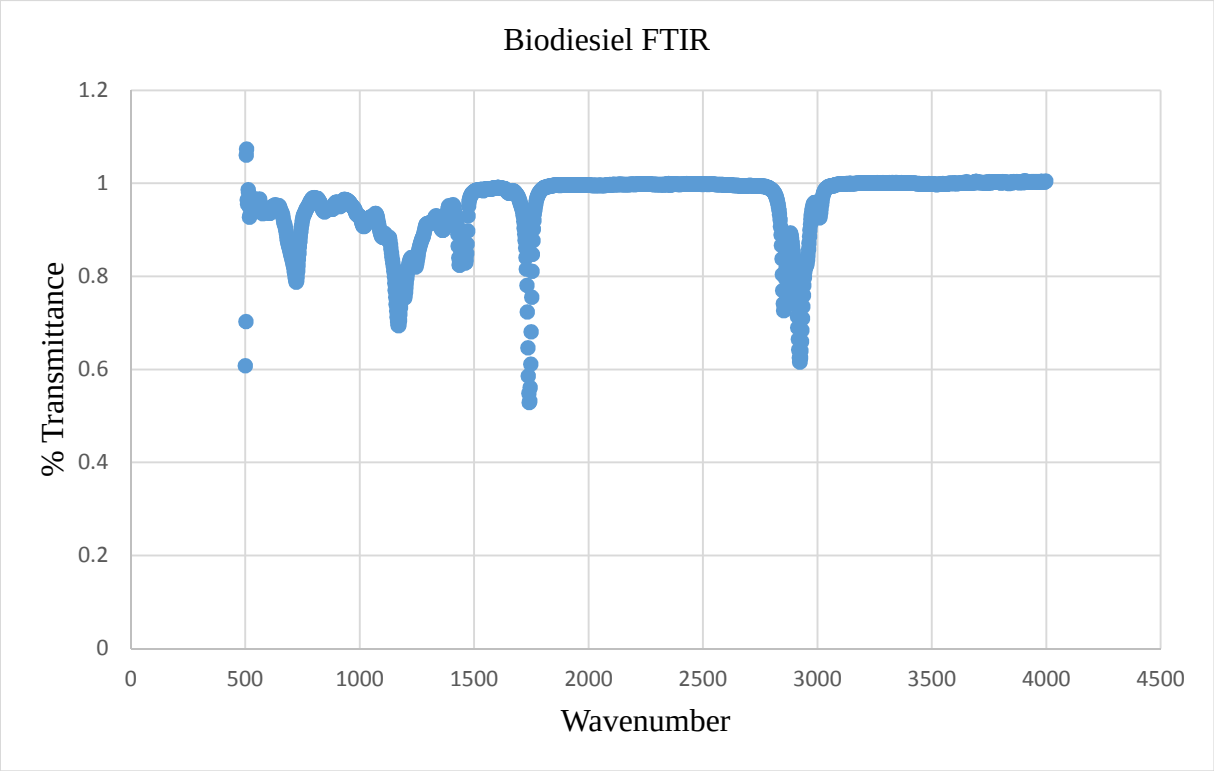

Fig 61: FTIR spectrum of Biodiesel

Interpretation of Biodiesel FTIR spectrum

1. The FTIR signal at 2926 cm^{-1} (rather than 1740 cm^{-1}) indicates presence of methyl esters, which means that the triacylglycerides are converted to free fatty methyl esters on transesterification reaction

2. The pear near 1500 is ester group indicator

3. The peak at 1300 wavenumber indicates the presence of Carboxylic group

4. The sharp peak near 1490 and 1700 is sodium hydroxide, this indicates that the biodiesel is not completely free from base catalyst sodium hydroxide.

5. The peak near 600 to 700 indicates c-o-c group

Interpretation of Diesel FTIR spectrum

Diesel and biodiesel FTIR spectrum had same peaks at 1500 and 3000 wave number, it indicates the biodiesel is produced from algal lipid transesterification .The Transmittance peaks in Diesel FTIR spectrum near 1500 wavenumber represent the aromatic compounds, the peak near 3000 represents alkane compounds present in the diesel(http://www.hindawi.com/journals/bmri/2011/196565/fig4/)

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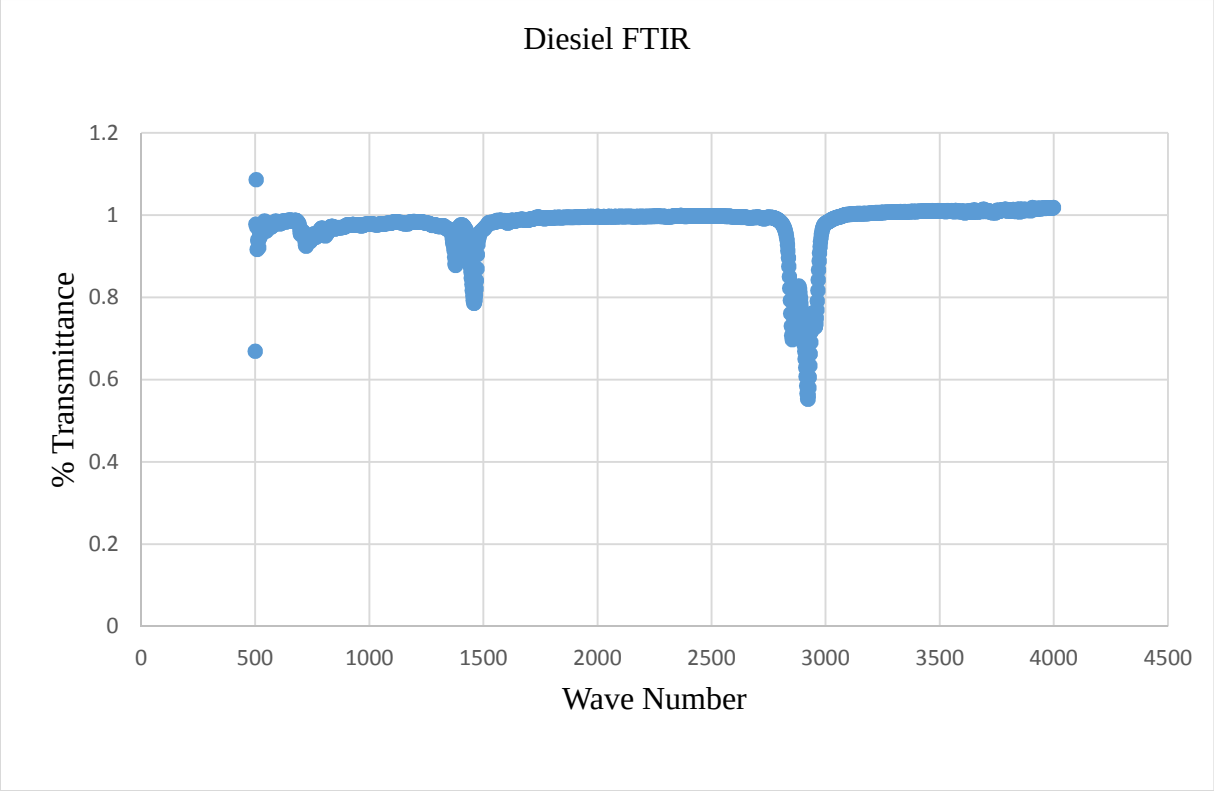

Fig62: FTIR spectrum of Diesel

From the FTIR spectrum analysis of biodiesel it as clear that the free fatty methyl esters are formed but the biodiesel still contained some unreacted sodium hydroxide as well as fatty acids , which did not undergo transesterification reaction. It can be concluded that biodiesel was produced from transesterification of algal lipid extracts but some amount of sodium hydroxide traces and fatty acids were present in biodiesel. No traces of methanol was observed, because methanol was completely removed during distillation of biodiesel after the transesterification reaction.

4.14 Material Balance

1. Specific Growth rate of algae in batch culture is 0.071 hr^{-1}
2. Lipid productivity from biomass was 0.0583 g/h
3. Yield of Lipids from lipid extraction was 19.6%
4. Yield of Biodiesel conversion by transesterification was 65%

SUMMARY AND CONCLUSION

5. Summary and Conclusion

The study of algae from different water sources in and around Rourkela has revealed that the *Cladophora Sp algae* is capable of rapid growth without maintaining any sophisticated culture conditions. In the pretreatment study it is witnessed that this algae has large amount of polysaccharides but very less water solubility and on different pretreatment methods it is found that Novozyme pretreatment followed by 0.2N HCl pretreatment and pretreatment with *Aspergillus Niger and T Reesei* was best suitable method for algae saccharification. The biodiesel conversion yield was 65% and 16% ethanol was produced from the fermentation. The biodiesel was flammable and has shown similar peaks corresponding to diesel in FTIR spectrum near 1490, 2900 range.

From this result, it is concluded that the *Cladophora Sp* is fastest growing algae and 19.8 % extractable lipids present in it. Among all axenic cultures it is a suitable strain to scale up in Rourkela. Production of 16% ethanol from fermentation indicating that simultaneous saccharification and fermentation can produce good yield of ethanol.

The Lipid yield of 19.8% indicates that the *cladophora sp* is fast growing and low oil containing algae. The 65% biodiesel conversion indicates that simultaneous production of biodiesel and ethanol is possible from same algae biomass. Furthermore, the oil yield can be improved by bringing the culture to steady phase and deprive nitrogen from media and use supercritical extraction and transesterification techniques for the biodiesel production

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